

APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: PROSTHESES WITH ASSOCIATED GROWTH FACTORS

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PROSTHESES WITH ASSOCIATED GROWTH FACTORS

Field of the Invention

5 The invention relates to prostheses having components that have been modified with a polypeptide growth factor. The invention further relates to methods for producing these prostheses.

Background of the Invention

10 Prostheses, i.e., prosthetic devices, are used to repair or replace damaged or diseased organs, tissues and other structures in humans and animals. Prostheses must be generally biocompatible since they are typically implanted for extended periods of time. For example, prostheses can
15 include artificial hearts, artificial heart valves, ligament repair material, vessel repair, surgical patches constructed of mammalian tissue and the like.

 Prostheses can be constructed from natural materials such as tissue, synthetic materials or a combination
20 thereof. For example, synthetic prostheses such as mechanical heart valve prostheses are manufactured from biocompatible metals and other materials such as graphite and polyester. Although mechanical heart valves have the advantage of proven durability through decades of use, they
25 are associated with a high incidence of blood clotting on or around the prosthetic valve. Blood clotting can lead to acute or subacute closure of the valve or associated blood vessel. For this reason, patients with implanted mechanical heart valves remain on anticoagulants for as long as the
30 valve remains implanted. Anticoagulants impart a 3-5%

annual risk of significant bleeding and cannot be taken safely by certain individuals.

Besides mechanical heart valves, heart valve prostheses can be constructed with tissue leaflets or
5 polymer leaflets. Thrombosis and subsequent calcification are concerns associated with polymer heart valves. Calcification of these valves can lead to failure.

Prosthetic tissue heart valves can be derived from, for example, porcine heart valves or manufactured from other
10 biological material such as bovine pericardium. Biological materials in prosthetic heart valves generally have profile and surface characteristics that generally provide laminar, nonturbulent blood flow. Therefore, intravascular clotting is less likely to occur than with mechanical heart valves.
15 Unfortunately, prosthetic tissue heart valves are limited by a tendency to fail beginning about seven years following implantation. Valve degeneration is particularly rapid in young patients and during pregnancy.

Calcification, i.e., the deposition of calcium
20 salts, especially calcium phosphate (hydroxyapatite), appears to be a major cause of degeneration. Efforts to address the calcification problem have included treating glutaraldehyde-fixed valve prostheses with compounds to reduce calcium nucleation. Other approaches include use of
25 alternative tissue fixation techniques since evidence suggests that glutaraldehyde fixation can contribute to calcification and mechanical degradation. In addition, since nonviable cells can be sites for calcium deposition, various processes have been developed to remove nonviable
30 cells while leaving the extracellular matrix intact. Intact tissue with viable cells has natural protection against calcification.

Another major disadvantage of tissue based prostheses is the failure of such devices to be self-maintaining. Long term durability is affected by the ability of viable cells to populate the implanted tissue and to carry out maintenance functions. The importance of viable cells has been studied in the context of allograft transplants, i.e., transplants from one member of a species to another member of the same species. Proper allograft preservation can maximize the number of viable cells remaining in the tissue as determined by matrix protein synthesis. Preservation techniques that do not promote cell survival, such as long term storage at 4°C, are associated with reduced *in vivo* durability and increased reoperation rates.

Summary of the Invention

A polypeptide growth factor can be joined with a tissue substrate or a synthetic substrate to promote population of the substrate with viable cells. Preferred polypeptide growth factors include vascular endothelial growth factors (VEGF). With crosslinked tissue, associated VEGF alleviates at least some of the cellular toxicity resulting from glutaraldehyde crosslinking. The VEGF can be joined with the substrate by direct contact in solution. Alternatively, the VEGF can be joined with the substrate either through application to the substrate along with a binder or through chemical binding of the VEGF to the substrate with or without an intervening linker molecule.

A substrate modified with VEGF provides for affiliation of viable endothelial cells with the substrate to improve the performance of the substrate as a prosthesis. For example, the long term durability of the prosthesis should be improved in part due to a reduction in

calcification, and the incidence of infection associated with the prosthesis should be reduced due to a decrease of locations suitable for the attachment of microorganisms. Using a cell culture system, the VEGF treated substrate can promote in vitro population of the substrate with endothelial cells. In addition, the VEGF can promote population of the substrate with endothelial cells in vivo following implantation of the substrate as part of a prosthesis.

In a first aspect, the invention features a prosthesis for a human patient comprising allograft, xenograft or synthetic tissue having a polypeptide growth factor joined therewith, the polypeptide growth factor being effective to stimulate the affiliation of viable cells with the tissue. The binding of the polypeptide growth factor to the tissue can involve specific binding interactions and/or covalent bonding. The binding of the polypeptide growth factor to the tissue can involve a linker molecule.

The tissue can include crosslinked tissue and/or uncrosslinked tissue. The tissue can be derived from porcine heart valves, bovine pericardial tissue, or any other synthetic or biological material. The polypeptide growth factor can include vascular endothelial growth factor. Suitable vascular endothelial growth factors include, for example, a protein selected from the group consisting of bVEGF164, bVEGF120, hVEGF165, hVEGF121, VEGF II, hVEGF80, VEGF-B, VEGF2, modified active forms thereof, and combinations thereof.

In another aspect, the invention features an article including crosslinked tissue with associated VEGF. The crosslinking can involve glutaraldehyde moieties.

In another aspect, the invention features a prosthetic heart valve comprising associated VEGF. The prosthetic heart valve can include a porcine heart valve.

In another aspect, the invention features a method of producing a prosthesis for a human patient, the prosthesis including allograft or xenograft tissue, the method including binding polypeptide growth factor to the tissue. The method further can include incubating the tissue having bound polypeptide growth factor with viable cells in vitro to affiliate the cells with the tissue. The cells can include human cells. The cells can include cells obtained from an intended recipient of the prosthesis.

In another aspect, the invention features a method of modifying a substrate, the method including incubating viable cells in vitro with tissue to affiliate the cells with the substrate, the substrate including associated polypeptide growth factor.

Other features and advantages of the invention are apparent from the following detailed description of the invention and from the claims.

Brief Description of the Drawings

Fig. 1 is a photograph of a crosslinked tissue sample that was treated with VEGF prior to a five day incubation during which time the tissue was in contact with viable endothelial cells grown on an insert within a microtitre well. Endothelial cells present on the fixed tissue are visualized by fluorescent labeling.

Fig. 2 is a photograph of a crosslinked tissue sample that was treated with VEGF prior to a five day incubation with endothelial cells in a microtitre well. In this example, the tissue treated with VEGF was not in direct contact with endothelial cells at the start of the

incubation. Endothelial cells present on the fixed tissue are visualized by fluorescent labeling.

Detailed Description of the Invention

A polypeptide growth factor or a fragment thereof
5 can be associated with a tissue substrate or a synthetic
substrate in vitro. Generally, the substrate forms, or will
form, a portion of a prosthesis. Preferred polypeptide
growth factors include vascular endothelial growth factors
(VEGF). Following modification of the substrate with VEGF,
10 the VEGF can stimulate endothelial cell chemotaxis and
proliferation. In preferred embodiments, the substrate is
fixed. The affiliation of viable endothelial cells with the
prosthetic tissue should contribute to the long term
viability of the prosthesis. VEGF modification is
15 particularly suitable for the production of prostheses that
naturally have an endothelial or epithelial cell lining,
such as vascular components, cardiovascular elements,
portions of the lymphatic system, uterine tissue or retinal
tissue.

20 The VEGF can be joined with the tissue in a variety
of ways. For example, the tissue can be combined with a
VEGF solution such that the VEGF becomes joined with the
prosthetic tissue by direct attachment. Alternatively, the
VEGF can be associated with the prosthetic tissue using an
25 adhesive. In addition, the VEGF can be joined with the
prosthetic tissue using chemical bonding.

The VEGF can effectively induce the growth of
endothelial cells on the tissue in vitro or in vivo such
that the tissue becomes populated with viable cells. For in
30 vivo growth, the tissue with associated VEGF can be
implanted into a patient. Once implanted in the patient,
endothelial cells are attracted to the prosthetic tissue due

to the presence of the VEGF. Alternatively, endothelial cells can be associated with the prosthetic tissue in a cell culture system, as described below.

A. Prostheses

Prostheses can include a tissue substrate or a synthetic substrate at least as a component, such that the substrate is suitable as a location for cellular attachment. Generally, these prostheses are designed for implantation into or onto a patient for extended periods of time.

Prostheses include, for example, artificial hearts, artificial heart valves, annuloplasty rings, stents, vascular grafts, dermal grafts for wound healing, and surgical patches constructed of mammalian tissue.

Natural tissues for use as substrates are derived from an animal species, typically mammalian, such as human, bovine, porcine, seal or kangaroo. These tissues can be obtained from, for example, heart valves, aortic roots, aortic walls, aortic leaflets, pericardial tissue such as pericardial patches, connective tissue such as dura mater, bypass grafts, tendons, ligaments, skin patches, blood vessels, human umbilical tissue, bone and the like. These natural tissues generally include collagen-containing material. Natural tissue is typically, but not necessarily, soft tissue. A tissue-based prosthesis can maintain structural elements from its native form, and/or structural elements can be incorporated into the prosthesis from the assembly of distinct pieces of tissue. For example, a heart valve prosthesis can be assembled from a porcine heart valve, constructed from bovine pericardium or a combination thereof.

Synthetic substrates can be formed from synthetic polymers and/or biological polymers, such as those generally

found in a natural tissue matrix, to form a synthetic tissue matrix. In particular, collagen and elastin polymers can be formed into a matrix corresponding to a tissue component by any of a variety of techniques such as weaving and molding.

5 The synthetic substrate formed from these biological polymers mimic a natural tissue matrix. Alternatively, synthetic substrates can be in the form of a synthetic tissue with a matrix including synthetic and/or biological polymers along with viable and/or non-viable cells.

10 Suitable synthetic and biological polymers are described below.

Tissues can be fixed by crosslinking. This provides mechanical stabilization, for example, by preventing enzymatic degradation of the tissue. Crosslinking also
15 removes antigenic sites that could result in the patient's rejection of the prosthesis. Glutaraldehyde typically is used for fixation, but other fixatives can be used, such as epoxides, formaldehyde and other difunctional aldehydes. Xenografts, i.e., prostheses incorporating tissue from a
20 species different from the patient's species, generally are fixed prior to use. Allografts, i.e., prostheses incorporating tissue of a different individual of the patient's species, can be fixed prior to use.

The prostheses can include other non-tissue
25 components such as polymeric material, ceramics and metal. Appropriate ceramics include, without limitation, hydroxyapatite, alumina and pyrolytic carbon. Polymeric materials can be fabricated from synthetic polymers as well as purified biological polymers. Appropriate synthetic
30 materials include hydrogels and other synthetic materials that cannot withstand severe dehydration.

Appropriate synthetic polymers include without limitation polyamines (e.g., nylon), polyesters,

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polystyrenes, polyacrylates, vinyl polymers (e.g., polyethylene, polytetrafluoroethylene, polypropylene and poly vinyl chloride), polycarbonates, polyurethanes, poly dimethyl siloxanes, cellulose acetates, polymethyl
5 methacrylates, ethylene vinyl acetates, polysulfones, nitrocelluloses and similar copolymers. Bioresorbable polymers can also be used such as dextran, hydroxyethyl starch, gelatin, derivatives of gelatin, polyvinylpyrrolidone, polyvinyl alcohol, poly[N-(2-
10 hydroxypropyl)methacrylamide], poly(hydroxy acids), poly(epsilon-caprolactone), polylactic acid, polyglycolic acid, poly(dimethyl glycolic acid), poly(hydroxy buterate), and similar copolymers. These synthetic polymeric materials can be woven into a mesh to form a matrix or substrate.
15 Alternatively, the synthetic polymer materials can be molded or cast into appropriate forms.

Biological polymers can be naturally occurring or produced *in vitro* by, for example, fermentation and the like. Purified biological polymers can be appropriately
20 formed into a substrate by techniques such as weaving, knitting, casting, molding, extrusion, cellular alignment and magnetic alignment. For a description of magnetic alignments see, for example, R. T. Tranquillo et al., *Biomaterials* 17:349-357 (1996), incorporated herein by
25 reference. Suitable biological polymers include, without limitation, collagen, elastin, silk, keratin, gelatin, polyamino acids, cat gut sutures, polysaccharides (e.g., cellulose and starch) and copolymers thereof.

B. Vascular Endothelial Growth Factor (VEGF)

30 VEGF refers to a family of polypeptides that have been found to preferentially stimulate growth of vascular endothelial cells over other cells such as smooth muscle

cells. Several forms of VEGF have been identified. VEGF polypeptides generally have sequence homology with platelet-derived growth factor, which can alter the migration and proliferation of a variety of cell types. VEGF occasionally
5 has been referred to as vascular permeability factor.

The originally identified form of VEGF has a molecular weight of about 46 kilodaltons (kDa). This form apparently is a homodimer with each subunit having a molecular weight of about 23 kDa. The c-DNA sequences
10 encoding the human polypeptide (165-amino acids, hVEGF₁₆₅) and the corresponding bovine polypeptide (164-amino acids, bVEGF₁₆₄) have been determined. In addition, variants of the polypeptides with 121-amino acids for the human version (hVEGF₁₂₁) and 120-amino acids for the bovine version
15 (bVEGF₁₂₀) also have been identified. For the corresponding amino acid sequences, see U.S. Patent 5,194,596, to Tischer et al., incorporated herein by reference. Other insoluble variants have been identified with 189 and 206-amino acids respectively. See, for example, E. Tischer et al., "The
20 human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing," J. Biol. Chem. 266:11947-11954 (1991) and K. A. Houck et al., "The vascular endothelial growth factor family: identification of a fourth molecular species and
25 characterization of alternative splicing of RNA," Molec. Endocrinology 5:1806-1814 (1991), both incorporated herein by reference.

Another form of VEGF, entitled VEGF II, is a heterodimer. As isolated from rat glioma cells, the first
30 subunit has 190-amino acids while the second subunit has a 135-amino acid form and an 115-amino acid form. VEGF II is described in EP 0 476 983A, incorporated herein by reference.

A single polypeptide human VEGF, unnamed, also has been identified. This polypeptide has a molecular weight of roughly 80 kDa. The corresponding cDNA was isolated and a 728-amino sequence was determined from the cDNA sequence.

5 Details of the protein are provided in EP 0 550 296A, incorporated herein by reference.

Still another human growth factor, VEGF2, has been identified from early stage human embryo osteoclastomas, adult heart and several breast cancer lines. VEGF2 has 350
10 amino acids, of which about 24 amino acids represent a leader sequence. The sequence for VEGF2 is disclosed in WO 95/24473, incorporated herein by reference.

Recently, VEGF-B, another variant of VEGF, has been identified. VEGF-B appears to be associated with heart and
15 skeletal muscles. Full sequences for mouse and human VEGF-B are presented in U.S. Patent 5,607,918, to Eriksson et al., incorporated herein by reference.

As described above, a variety of VEGF polypeptides have been identified. Many of these are associated with
20 particular tissues. At least some of the polypeptides have variations based on alternative message splicing, such as hVEGF₁₆₅ and hVEGF₁₂₁. As used in the other sections of this application, "VEGF" refers to all previously identified VEGF polypeptides, such as those described in this section, as
25 well as any future identified VEGF polypeptides, that selectively promote the chemotaxis or proliferation of endothelial cells. "VEGF" also refers to polypeptide fragments that maintain their ability to selectively promote the chemotaxis or proliferation of endothelial cells. As
30 noted above, for example, human VEGF₁₂₁ is a naturally occurring fragment of human VEGF₁₆₅. Recombinant human VEGF₁₆₅, human VEGF₁₂₁, and mouse VEGF are available from R&D Systems of Minneapolis, MN.

Using standard molecular biology techniques (see, for example, Sambrook, Fritsch and Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd edition, Cold Spring Harbor Press, (1989)), it is possible to make recombinant modified forms of natural VEGF polypeptides. These straightforward modifications include addition of amino acids on the N-terminus, the C-terminus or both. Also, modifications can be made by substituting amino acids along the polypeptide chain. Some modifications may destroy activity of the enzyme. It is straightforward to eliminate inactivating modifications by testing for activity in cell culture systems. Active forms of these modified polypeptides are within our general definition of "VEGF."

C. Joining of VEGF with a Substrate

The joining of VEGF with a substrate can involve direct attachment, application of a coating including an adhesive, or chemical binding. Direct attachment entails combining the substrate with a solution of the VEGF. It has been discovered that the VEGF can associate with biological tissue such that it is not easily washed off. The binding of the VEGF to fixed tissue seems to last for at least moderate periods of time, up to at least several days, when the tissue is in contact with a solution. Evidence has been obtained, as set forth in the Example below, that treatment with ethanol prior to contact with the VEGF reduces the association of VEGF with fixed tissue. The reduction of the association of VEGF resulting from rinsing the tissue with ethanol possibly could be due to elimination of VEGF binding sites, inactivation of VEGF binding sites or binding of ethanol at VEGF binding sites.

For direct binding of VEGF to a substrate, the substrate or a portion thereof is combined with a solution

of the VEGF at a concentration generally from about 1ng/ml to about 1μg/ml and preferably from about 25ng/ml to about 250ng/ml. During incubation with the VEGF, the solution preferably is cooled, for example, to about 4°C. The substrate preferably remains in the VEGF solution at about 4°C for about 24 hours and up to about 14 days or more. The VEGF solution preferably is buffered at a pH ranging from about 6 to about 8.5, and more preferably ranging from about 6.3 to about 7.4. Suitable buffers can be based on, for example, the following compounds: phosphate, borate, bicarbonate, carbonate, cacodylate, citrate, and other organic buffers such as tris(hydroxymethyl)aminomethane (TRIS), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), and morpholine propanesulphonic acid (MOPS).

Alternatively, VEGF can be associated with the substrate through the use of a binder or adhesive. The VEGF and the adhesive form a coating on the substrate. Preferred adhesives include, for example, biologic glues such as fibrin glue, and the like. Fibrin glue can be formed from the polymerization of fibrinogen and thrombin. Suitable fibrin glues are available from, for example, Immuno AG, Austria and Zymogenetics, Seattle, WA. A variety of similar glues are known, such as an adhesive based on fibrinogen, factor XIII, and albumin (see U.S. Patent 4,414,976, to Schwarz et al., incorporated herein by reference) and an adhesive based on water soluble proteinaceous material and polyaldehydes (see U.S. Patent 5,385,606, to Kowanko, incorporated herein by reference).

To apply the VEGF with a fibrin glue, a small amount of thrombin can be absorbed to the substrate. VEGF can be mixed with a solution containing fibrinogen, to yield a solution with a VEGF concentration preferably ranging from about 1ng/ml-10μg/ml. Then, the fibrinogen/VEGF mixture can

be brushed over the surface of the substrate with absorbed thrombin, or the tissue with absorbed thrombin can be dipped into the fibrinogen/VEGF solution. The VEGF-adhesive coating can be applied to all or just a portion of the substrate. With synthetic substrates, the VEGF also can be incorporated into the substrate material when the substrate is formed.

In other embodiments, the association of VEGF with the substrate involves chemical binding. Chemical binding includes, for example, covalent bonding, a plurality of noncovalent chemical interactions or both covalent and noncovalent interactions. Noncovalent chemical interactions include, for example, hydrogen bonding, van der Waals interactions and molecular rearrangements, which characterize, for example, antibody-antigen, specific binding protein-receptor and enzyme-substrate associations. In other words, reactants or binding agents are used to form a direct chemical interaction between the VEGF and the substrate, possibly involving a linker molecule. The chemical binding of the VEGF preferably takes place at or near a physiological pH, preferably ranging from about 6 to about 8.5 and more preferably from about 6.3 to about 7.4.

The chemical binding of VEGF can involve covalent bonding to the surface of the substrate with reactive agents such as glutaraldehyde and other general crosslinking agents. A typical procedure for general binding of VEGF to the surface of a tissue makes use of glutaraldehyde, which crosslinks proteins by way of two aldehyde groups. Since glutaraldehyde is typically used for fixation of some biocompatible materials, the non-specific crosslinking to bind the VEGF to the biocompatible material can be performed simultaneously with fixation of the tissue. Alternatively, the non-specific crosslinking to covalently bond the VEGF

can be performed as a separate step before or after the completion of a fixation process, assuming a fixation step is performed. Other chemical reagents for covalent bonding of VEGF to a substrate include, for example, epoxies.

5 On the other hand, chemical binding of the VEGF to the substrate can involve specific binding interactions. If selected accordingly, the specific binding interactions can be used to target specific locations within the substrate. The targeting of particular locations can be useful, for
10 example, if particular locations are resistant to colonization by endothelial cells or if colonization by endothelial cells is particularly beneficial at particular locations. Examples of possible target location include heart valve leaflets.

15 One method of targeting a particular location involves the use of linkers that target specific cellular or extracellular binding sites within a natural tissue. In certain embodiments, the linker is covalently bound to the VEGF molecule, and the linker associates with the tissue by
20 a plurality of non-covalent interactions. Alternatively, the linker can be covalently bound to the tissue and the VEGF can be associated with the linker by a plurality of non-covalent interactions. A variety of commercially available antibodies and other specific binding reagents may
25 be used as linkers. Alternatively, antibodies can be prepared by conventional techniques.

 A VEGF polypeptide having an attached antibody or any other comparable targeting molecule or an engineered chimera of the VEGF polypeptide and the targeting molecule
30 is considered a VEGF molecule for the purposes of the present application. The chemical binding of compounds to antibodies as well as the development of chimeras is well established, especially where the compound is a protein.

Empirical adjustments can be made to ensure that the activity of the VEGF molecule is not significantly impaired.

In an alternative embodiment, photochemical coupling can be used for covalent coupling. Photochemical coupling is based on the use of high energy light, e.g., ultraviolet light, to form reactive intermediates of certain functional groups. These reactive intermediates can form carbon-carbon bonds between two compositions. Aryl ketone functional groups are particularly useful in this respect.

Photochemical coupling can be used for attachment of VEGF to tissue. See, for example, Dunkirk et al., J. Biomaterials Applications 6:131-156 (1991), incorporated herein by reference. The tissue may or may not be separately crosslinked since the photochemical coupling generally also crosslinks the tissue, i.e., photofixation. Alternatively, photochemical coupling can be used to attach a linker to the tissue either before, after, or during binding of the linker to the VEGF polypeptide.

Regardless of the nature of the interaction, the bound VEGF generally is in equilibrium with unbound molecules. As a result, the VEGF may eventually be lost to the surrounding solution if the solution is replenished. For some applications it may be sufficient for the VEGF to be bound for a relatively short period of time such as hours or days if sufficient viable endothelial cells proliferate on the tissue during the relevant time. In other circumstances, it may be desirable for longer term binding of the VEGF to the tissue such as months or years. The nature of the association of the VEGF with the tissue can be selected accordingly.

D. Other Modifiers

It may be desirable to associate other chemicals with the substrate, in addition to the VEGF, to improve its performance in a prosthesis. As noted above,

endothelialization due to joining of VEGF with the a
5 substrate generally reduces incidence of calcification and infection. Nevertheless, it may be desirable to include chemicals that act to further reduce calcification and/or microbial infection. For example, aluminum, iron and magnesium ions have been found to reduce calcification.

10 These polyvalent ions can be directly associated with tissue as described in U.S. Patent 5,094,661, to Levy et al., incorporated herein by reference.

~~ANSF~~ Alternatively, the polyvalent ions can be associated with exogenous storage structures which are in turn
15 associated with the substrate. The use of exogenous storage structures for the storage of anticalcification metal ions is described in copending, commonly assigned patent applications Serial Nos. 08/595,402 and 08/690,661, both incorporated herein by reference. Similarly, certain metals
20 such as silver have been associated with antimicrobial activity. Exogenous storage structures can be used to store suitable antimicrobial metal ions in association with a substrate as described in copending and commonly assigned patent application Serial No. 08/787,139, incorporated
25 herein by reference. Preferred exogenous storage structures include, for example, ferritin and other metal storage proteins. The exogenous storage proteins can be associated with the substrate in ways similar to those used for VEGF. The activities should not interfere with each other.

30 E. In vitro Attachment of Endothelial Cells

Growth of viable endothelial cells on prostheses prior to implantation into a patient can be promoted in

)
vitro by joining VEGF with a substrate. In order to reduce the possibility of transplant rejection, the endothelial cells used for in vitro endothelialization preferably are autologous cells, i.e., cells from the ultimate recipient.

5 Suitable cells could be harvested from, for example, adipose tissue of the patient. The harvesting process can involve liposuction followed by collagenase digestion and purification of microvascular endothelial cells. A suitable process is described further in S. K. Williams, "Endothelial
10 Cell Transplantation," Cell Transplantation 4:401-410 (1995), incorporated herein by reference and in U.S. Patents 4,883,755, 5,372,945 and 5,628,781, all three incorporated herein by reference. Purified endothelial cells can be
15 suspended in an appropriate growth media such as M199E (e.g., Sigma Cell Culture, St. Louis, MO) with the addition of autologous serum.

Prosthetic tissue with bound VEGF can be incubated in a stirred cell suspension for a period of hours to days to allow for endothelial cell seeding. Cell seeding
20 provides random attachment of endothelial cells that can proliferate to coat the surface of the prosthetic substrate either before or after implantation into the patient. Alternatively, the prosthetic substrate can be incubated under a pressure gradient for a period of minutes to promote
25 cell sodding. A suitable method for cell sodding can be adapted from a procedure described for vascular grafts in the S. K. Williams article, supra. Cell sodding can produce a monolayer of cells on the surface of the prosthetic tissue.

30 In addition, the prosthetic tissue can be placed in a culture system where the patient's endothelial cells are allowed to migrate onto the surface of the prosthetic substrate from adjacent plastic tissue culture surfaces. If

either attachment or migration of endothelial cells is performed under conditions involving physiological shear stress, then the endothelial cells colonizing the surface of the substrate may express appropriate adhesion proteins that
5 allow the cells to adhere more tenaciously following implantation.

F. Storage, Packaging, Distribution and Use

Following binding of the VEGF to the substrate, the substrate, possibly formed into a prosthesis, can be stored.
10 The substrate preferably would not have ingrowth of viable cells if the substrate is intended for longer storage. Preferred storage techniques minimize the risk of microbial contamination. For example, the biocompatible material can be stored in a sealed container with sterile buffer and/or
15 saline solution.

In a sealed container the biocompatible material is not subjected to a continuous supply of fluids. Nevertheless, consideration should be given to possible loss of VEGF or VEGF activity from the substrate during storage.
20 If excessive loss is a possibility, the storage time can be limited appropriately to keep the loss to an acceptable level. For distribution, the prostheses generally are placed in sealed and sterile containers. The containers can be dated such that the date reflects the maximum advisable
25 storage time accounting for possible loss or degradation of VEGF activity. The containers are distributed to health care professionals for surgical implantation of the prostheses. In vitro affiliation of cells with VEGF modified prosthesis preferably is performed at hospitals
30 where the patient's cells can be removed for use in a cell culture system.

As an alternative to the above storage and distribution approach, the VEGF modification can be performed at a hospital, if desired. Then, the prosthesis prepared for VEGF modification is distributed. Once the
5 prosthesis is modified with VEGF, it can be implanted, stored for a reasonable period of time or introduced into a cell culture system to affiliate autologous cells with the VEGF modified prosthesis.

Incorporation of VEGF into a prosthesis to promote
10 endothelialization of a substrate should improve biocompatibility of the substrate following implantation. In particular, a quiescent endothelial cell monolayer can serve as a barrier to infection, inflammation, and calcification. Endothelialization of a prosthesis also can
15 promote further recellularization of the prosthesis with cells capable of repairing and remodeling the tissue. Thus, the durability and the longevity of a prosthesis can be significantly improved. Ultimately, recellularization can provide for a prosthesis that more closely resembles a
20 native, biologically competent tissue.

Example 1 - Direct VEGF Association

This example demonstrates the ability of VEGF to associate with crosslinked tissue and the corresponding effectiveness of the VEGF to stimulate affiliation of viable
25 endothelial cells with the tissue.

Several solutions were prepared. The glutaraldehyde solution was prepared in a 5 liter volume by the addition of 19.3g NaCl, 70.0g sodium citrate, 2.5g citric acid, 50ml of 50% by weight glutaraldehyde, and sufficient reverse osmosis
30 purified water (RO water). A VEGF solution was prepared by diluting 50 μ g/ml stock solution of VEGF (human recombinant VEGF₁₆₅, R&D Systems, Minneapolis, MN) with 5ml of HBSS (30mM

HEPES buffered saline solution from Clonetics, San Diego, CA) for a final concentration of 100ng/ml. A HEPES buffered saline solution was prepared by adding 17.4g of NaCl and 35.7g HEPES free acid to three liters of RO water. An 80% ethanol solution was prepared by combining 1.8g NaCl, 3.8g HEPES free acid, 1684mls of 95% by weight ethanol and 316mls of RO water to make 2 liters of solution. All solutions were sterile filtered prior to use.

To prepare the samples, 75 porcine heart valve leaflets were removed from harvested porcine heart valves. The leaflets were stored overnight at 4°C in 0.9% sterile saline. Then, the leaflets were glutaraldehyde crosslinked in citrate buffered glutaraldehyde solution for a minimum of 6 days. The glutaraldehyde solution was changed twice during the crosslinking procedure, after 24 hours and after three days. The crosslinked leaflets were stored in HEPES buffered glutaraldehyde at room temperature either for 5 days followed by treatment with ethanol or for 46 days.

Then, 35 leaflets were removed from the glutaraldehyde and were treated with ethanol. Following removal from the glutaraldehyde, these leaflets were incubated in 500 ml of HEPES buffered saline for 10 minutes. This saline was poured off, and the leaflets were incubated in 500 ml of fresh HEPES buffered saline for an additional 15 minutes. After removal of the second saline solution, the leaflets were rinsed once with 80% ethanol and then soaked in 500 ml of 80% ethanol solution for 15 minutes. Then, the first ethanol solution was replaced with an equivalent 500 ml fresh 80% ethanol solution, and the leaflets were incubated in the second ethanol solution for about 24 hours at room temperature.

After 24 hours in ethanol, the leaflets were rinsed with HEPES buffered saline and then soaked in HEPES buffered

saline for 15 minutes. After changing the solution, the leaflets were soaked in HEPES buffered saline for 24 hours. The leaflets were then transferred to a storage container containing HEPES buffered saline. The leaflets in the storage container were subjected to gamma sterilization by SteriGenics (Charlotte, SC). Gamma irradiation caused the leaflets in HEPES buffered saline to turn brown. Following sterilization, the leaflets were stored in this container at 4°C for fifteen days until further use.

Both ethanol treated and non-ethanol treated leaflets were removed from storage and cut in half. The cut leaflets were rinsed three times with 100 ml of 0.9% sterile saline. Following the rinses, six of the ethanol treated and six of the non-ethanol treated leaflet halves were incubated in HBSS containing 100ng/ml VEGF. Leaflets were incubated in the VEGF solution overnight at 4°C.

Four six-well plates were prepared with 2% gelatin (Sigma Chemical, St. Louis, MO) and EGM media (Clonetics, San Diego, CA) to support cell growth. Human umbilical vein endothelial cells (HUVECs) from Clonetics (lot #2803) were grown to confluence in each of the 24 wells. Twenty four hours after achieving confluence, a sterilized rubber policeman was used to scrape the cells from the center of each well. Media containing the cellular debris was removed and replaced with fresh EGM media. Each well was examined by light microscopy to assure that cells have been removed from the center of each well.

Leaflet halves were placed in the scraped clear center of each plate, and either normal EGM media or EGM media containing 10ng/ml VEGF was added according to the following protocol:

- 1) No leaflet, normal media (3 wells);
- 2) No leaflet, VEGF in media (3 wells);

- 3) Ethanol treated leaflet, normal media (4 wells);
- 4) Ethanol treated leaflet, VEGF in media (4 wells);
- 5) Ethanol and VEGF treated leaflet, normal media (4 wells);
- 6) Non-ethanol treated leaflet, normal media (2 wells); and
- 7) VEGF treated leaflet, normal media (4 wells).

Leaflet halves that were not pretreated with VEGF were rinsed three times with sterile saline, as described previously. The VEGF pretreated leaflets had been rinsed before treatment with VEGF and did not receive any additional rinses before placement into a well.

A fifth six-well plate was used in which HUVECs were cultured onto the top membrane of tissue culture inserts that were placed inside each well. Once the cells on this membrane had achieved confluence, a hole was cut in the center of each insert membrane using a sterile scalpel. A leaflet was placed on the bottom of each well, and the insert was placed over the leaflet such that the edges of the hole in the insert membrane rested on the leaflet. Each well was filled with 2 mls of EGM media. On this plate, two of the leaflets were ethanol treated with no VEGF treatment, two leaflets had ethanol treatment followed by VEGF treatment and two leaflets had VEGF treatments but no ethanol treatment.

After about the first five hours, the media in all the wells of the five plates was replaced. The cells then were allowed to grow for a total of about five days with fresh media added every other day. After four days, all wells were examined using a light microscope. Since the leaflets are opaque, this technique did not allow for

visualization of cells attached to the leaflets. It was also impossible to see cells grown on top of the inserts since these membranes were also opaque. Given these limitations, the following observations were made:

- 5 1) Cells grown in wells containing no leaflets had resumed growth to cover the area scraped clear and were again almost confluent;
- 2) Most of the cells in the wells containing glutaraldehyde leaflets with no further
- 10 treatments were dead; and
- 3) Ethanol treated leaflets did not appear to be cytotoxic.

After five days, half of the tissue samples were rinsed twice with Dulbecco's phosphate buffered saline

15 (Gibco BRL, Grand Island, NY). The rinsed samples were fixed with 3% formaldehyde solution for at least five minutes. The fixed samples were rinsed three times with RO water and once with 0.25M sucrose.

A 5mM stock solution of a fluorescent, lipophilic

20 probe, dioctadecyl tetramethyl indocarbocyanine perchlorate (DiI) from Molecular Probes Inc., Eugene, OR (catalog No. D-282) was prepared by adding 0.00467 grams of DiI powder to 1ml of dimethyl sulfate (DMS) in a 1.5ml microcentrifuge tube. DiI is a cell membrane stain. The tube was vortexed

25 to dissolve the powder. The tube is stored at room temperature wrapped in aluminum foil and kept away from light sources. A 50 μ M solution of DiI was prepared by adding 150 μ l of the 5mM stock solution to 15mls of 0.25M sucrose solution in a centrifuge tube. The tube was

30 vortexed to mix the solution. The dilute DiI solution was made fresh on the day of use.

The tissue samples were fluorescently stained by covering each rinsed leaflet in its well with sufficient

50 μ M DiI solution. The plates were covered with aluminum foil to avoid light exposure. The leaflets were stained for at least about 15 minutes but no more than about 25 minutes. Then, the samples were rinsed four times with RO water.

5 Following rinsing, 0.9% saline was added to each sample to prevent it from drying out, and the samples were covered with aluminum foil to prevent bleaching prior to examination. The stained tissue samples were imaged using a tetramethylrhodamine isothiocyanate (TRITC) filter and
10 photographed.

No cells grew on the ethanol treated leaflets, and no cells grew on the untreated leaflets except for a few cells growing on one sample in contact with a membrane insert. Similarly, 10ng/ml VEGF in solution did not
15 stimulate the affiliation of cells with the tissue. Only background fluorescence was observed with leaflets lacking cells when examined through the TRITC filter. Leaflets with VEGF adsorbed to the surface had colonies of brightly fluorescent cells attached to the leaflets indicating
20 stimulation of endothelial cell migration toward the leaflet and of adherence to the leaflet. This can be seen in Fig. 1 for a representative leaflet in direct contact with endothelial cells on a membrane insert and in Fig. 2 for a representative leaflet placed in a section of a well
25 initially clear of endothelial cells. The use of an insert did not qualitatively alter the results.

The embodiments described above are intended to be illustrative and not limiting. Additional embodiments are within the claims.